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1) **INVOLVEMENT OF** CYCLOPHILIN - D **IN THE ACTIVATION OF A MITOCHONDRIAL PORE BY CA2+ AND OXIDANT STRESS** (Abstract Available)
Author(s): TANVEER A; VIRJI S; ANDREEVA L; TOTTY NF; HSUAN JJ; WARD JM; CROMPTON M
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2) **The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury.**
Halestrap A P
Department of Biochemistry, University of Bristol, U.K.
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3) **mport and processing of heart mitochondrial** cyclophilin D .
Johnson N; Khan A; Virji S; Ward J M; Crompton M
Department of Biochemistry and Molecular Biology, University College London, London, UK.
European journal of biochemistry / FEBS (GERMANY) Jul **1999** , 263 (2) p353-9, ISSN 0014-2956 Journal Code: 0107600

4) **The mitochondrial permeability transition pore and its role in cell death.**
Crompton M
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5) **The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins.**
Marzo I; Brenner C; Zamzami N; Susin S A; Beutner G; Brdiczka D; Remy R; Xie Z H; Reed J C; Kroemer G
Centre National de la Recherche Scientifique, Unite Propre de Recherche 420, F-94801 Villejuif, France.
Journal of experimental medicine (UNITED STATES) Apr 20 **1998** , 187 (8) p1261-71, ISSN 0022-1007 Journal Code: 2985109R

6) Cyclophilin - D **binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore.**
Crompton M; Virji S; Ward J M
Department of Biochemistry and Molecular Biology, University College London, UK. m.crompton@bsm.biochemistry.ucl.ac.uk
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Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore

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A cyclophilin-D affinity matrix was employed to isolate components of the mitochondrial permeability transition pore. A cDNA encoding cyclophilin-D was cloned from a rat liver library and ligated into pGEX to allow expression of a glutathione *S*-transferase/cyclophilin-D fusion protein in *Escherichia coli* XL1 cells. The cyclophilin-D in the fusion was functionally normal as judged by its peptidylprolyl *cis-trans*-isomerase activity and its inhibition by cyclosporin A. The fusion protein was bound to glutathione-agarose to form the cyclophilin-D affinity matrix. The matrix selectively bound 32-kDa proteins of mitochondrial membrane extracts, but no H_2O-soluble proteins were bound. The 32-kDa band on SDS/PAGE resolved into a doublet and reacted with antibodies against the voltage-dependent anion channel (porin) and the adenine nucleotide translocase. These two proteins were also selectively retained by the affinity matrix in the presence of cyclosporin A. The thus-purified voltage-dependent anion channel, adenine nucleotide translocase and the fusion protein were incorporated into phosphatidylcholine liposomes containing fluorescein sulphonate. The proteoliposomes were permeabilized by Ca^{2+} plus phosphate, and this was blocked completely by cyclosporin A. These properties are identical to those of the permeability transition pore in mitochondria. It is concluded that the basic permeability transition pore structure comprises the voltage-dependent anion channel (outer membrane), adenine nucleotide translocase (inner membrane) and cyclophilin-D, and forms at contact sites between the two membranes.

Keywords: Cyclophilin; permeability transition pore; adenine nucleotide translocase; porin.

Mitochondria contain a structure that forms a large pore in the inner membrane under conditions of high matrix [Ca^{2+}], P_i and oxidative stress ([1, 2], reviewed in [3]). Opening of the so-called permeability transition (PT) pore is fully reversed on Ca^{2+} removal [2]. Electrophysiological measurements have revealed that the pore flickers between open and closed states under the influence of Ca^{2+} [4, 5]. The internal diameter of the pore has been estimated to be about 2 nm [6], allowing free diffusion of most metabolites across the inner membrane, but not of proteins [7]. The physiological role of the PT pore has not been established. However, pore opening uncouples mitochondrial energy transduction and would compromise cell viability, and there is considerable interest in pore involvement in cell death. There are now numerous indications for PT pore involvement in cell necrosis brought on by oxidative stress and impaired Ca^{2+} homeostasis ([8–11] and references therein). In addition, the PT pore has been implicated in apoptosis ([12–14] and references therein). It has been proposed that mitochondrial swelling brought about by PT pore activation induces rupture of the outer membrane leading to the release of pro-apoptotic cytochrome *c* and apoptosis inducing factor from the intermembrane space [15]. There is evidence that the PT pore is controlled in an opposing manner by Bcl-2 [16] and Bax [14], proteins that localize to mitochondria and that are associated with the down-regulation and up-regulation of apoptosis, respectively.

The PT pore is blocked by cyclosporin A (CSA) [17], which binds to cyclophilin-D (CyP-D) resident in the matrix space [18–20]. Like other cyclophilins, CyP-D catalyzes rotation of prolylpeptide bonds in target peptides and proteins. The peptidylprolyl *cis-trans*-isomerase (PPIase) activity is inhibited by CSA, which binds to the active site of the enzyme. It has been assumed that PT pore opening entails the recruitment of CyP-D by a membrane protein with pore-forming properties and that CSA prevents such an interaction [18–20]. The adenine nucleotide translocase (ANT) has been regarded as a possible candidate for the PT pore and CyP-D interaction [18, 21]. Recently, pore activity has been reconstituted from protein fractions containing ANT and the voltage-dependent anion channel (VDAC, porin) of the outer membrane [22].

In the present study, we have produced a glutathione *S*-transferase (GST)–CyP-D fusion protein for the detection of CyP-D interacting proteins. The study shows for the first time that CyP-D binds specifically and tightly to a VDAC/ANT complex and that it does so even in the presence of CSA. When incorporated into proteoliposomes, purified VDAC, ANT and the fusion protein yield CSA-sensitive pore activity. A preliminary account of part of this work has been communicated [23].

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Abbreviations. PT, permeability transition; CSA, cyclosporin A; CyP-D, cyclophilin-D; ANT, adenine nucleotide translocase; VDAC, voltage-dependent anion channel; GST, glutathione *S*-transferase; PPIase, peptidylprolyl *cis-trans*-isomerase.

MATERIALS AND METHODS

cDNA cloning and expression of the GST–CyP-D fusion protein. A rat liver cDNA library in λ gt-11 was screened using

a 400-bp probe for CyP-D. The 400-bp fragment was generated by PCR from the same library using primers 5′-AACCCGCTC-GTGTACCTGGACGTG and 5′-CTTAGAGCCGAAAGATTC-TATTTT, corresponding to human CyP-D [24], and cloned in pCR-Script (Stratagene). The base sequence amplified between the primers was identical to that encoding amino acids 52-186 of rat CyP-D cDNA from a muscle library [25]. The fragment was labelled with [γ-^{32}P]dCTP using the RadPrime Labelling kit (Gibco). Positive clones obtained after screening approximately 10^5 recombinant phage were replated and rescreened, yielding four positive clones. After digestion with *Eco*R1, the longest insert (1.4 kbp) was cloned in pBluescript and sequenced at the ends. Apart from a single conservative base substitution (C for G at position 185), the sequences were the same as bases 68–233 (encoding amino acids 14–68) and 1020–1288 (3′ noncoding region) of rat CyP-D cDNA from muscle [25]. These embrace the entire coding region for mature CyP-D. To construct the expression vector, the coding sequence for mature CyP-D was PCR-amplified from the cloned cDNA in pBluescript using the primer pair 5′-CGCGGGATCCAGTGCAGCGACGGC-GGA and 5′-CGCGGAATTCTTAGCTCAACTGGCCACAGT; these correspond to the sequences encoding the N-terminus and C-terminus of the mature protein, with added *Bam*H1 and *Eco*R1 sites, respectively. The PCR product was ligated into pGEX-3X (Pharmacia), enabling CyP-D expression as an N-terminal GST fusion protein.

For expression, *Escherichia coli* XL1 cells transformed with the recombinant plasmid were cultured overnight at 37°C in 5 ml nutrient broth (Oxoid) containing 100 μg/ml ampicillin. The following day, 1 ml culture was diluted 10-fold with fresh medium. After 2 h, 0.5 mM isopropyl thio-β-D-galactoside was added to induce expression. The cells were harvested 4 h later and sonicated for 45 s in 100 mm NaCl, 10 mM Tris/HCl 0.5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, pH 7.2. Insoluble matter was sedimented (10000 *g*, 5 min) and the supernatant mixed with GSH immobilized on agarose beads (Sigma) for adsorption of the fusion protein. Routinely, about 500 μg fusion protein was produced from 10 ml induced culture, and this was applied to a 1 ml bed volume of GSH-agarose. The affinity matrix was then washed with at least 10 vol. 50 mM NaCl, 10 mM Hepes, pH 7.3, to remove non-adherent protein.

Control affinity matrices contained GST alone, in place of the GST–CyP-D fusion. These were prepared in the same way except that cells were transformed with non-recombinant pGEX-3X.

Binding of mitochondrial proteins to the affinity matrix. Rat heart mitochondria were prepared, and their protein contents determined, as described previously [6]. Mitochondria (about 100 mg protein) were sonicated (10 μm, 2 min) in 4 ml 50 mM NaCl, 10 mM Tris/HCl, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg×ml^{-1} pepstatin, 1 μg×ml^{-1} leupeptin, pH 7.2, and centrifuged (70000 *g*, 40 min). The supernatant was retained as the soluble fraction. The pellet was extracted with 4 ml of the same medium containing 100 μm ADP and 6% Chaps. After 30 min mixing on ice, extracts were clarified by centrifugation (20000 *g*, 20 min).

Binding to the affinity matrix was conducted in Eppendorf tubes. Each tube contained 40 μl affinity matrix slurry, 300 μl membrane extract or soluble fraction, and additions noted. After gentle mixing (1 min, room temperature) the tubes were spun briefly and the supernatants discarded. The affinity matrix was then washed several times, each time with 400 μl 50 mM NaCl, 10 mM Tris/HCl, pH 7.2, 0.5 mM dithiothreitol and (with membrane extracts) 0.6% Chaps; other additions are noted in the figure legends. The GST–CyP-D fusion protein (GST in control experiments) along with proteins bound to it were displaced with 50 mM GSH, then analysed by SDS/PAGE.

Reconstition and assay of the PT pore. The GST–CyP-D affinity matrix was loaded with membrane extract, washed six times, and residual proteins displaced with GSH as described above. The displaced proteins (containing about 5 μg VDAC plus ANT) were mixed with 10 mg phosphatidylcholine dissolved in 0.5 ml of 4% (mass/vol.) Chaps, 20 mM NaCl, 10 mM Tris/HCl, pH 7.4. The mixture was dialysed overnight against the same medium minus Chaps. The formed liposomes were mixed with 10 mM fluorescein sulphonate, sonicated briefly (5 s), and the proteoliposomes separated by passage (twice) through Sephadex G-25. For assay, the vesicles were diluted with an equal volume of 20 mM NaCl, 40 mM sucrose, 10 mM Tris/HCl, pH 7.4. Fluorescence was measured at 480 nm (excitation) and >510 nm (emission) in a 30-μl reaction chamber mounted on the stage of a fluorescence microscope (10× objective) equipped with a side mounted PM tube (Cairn Research).

Western blotting. Proteins separated by SDS/PAGE were transferred to nitrocellulose membranes (Schleucher and Schuell) in 25 mM Tris/HCl, 190 mM glycine, 20% methanol, pH 8.3. After blocking with 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, 0.1% Tween-20 (NaCl/P_i/Tween) containing 5% milk powder, the membranes were washed three times in NaCl/P_i/Tween. For VDAC, the membranes were then incubated in NaCl/P_i/Tween/1% milk powder containing 5 μg/ml anti-VDAC monoclonal antibody (Calbiochem). After 1 h, the membranes were washed five times in NaCl/P_i/Tween. Goat anti-mouse IgG coupled to horse radish peroxidase was used for detection. Bands were developed with the enhanced chemiluminescence (ECL) kit from Amersham. For ANT, membranes were decorated with anti-ANT polyclonal antibodies in the laboratory of Dieter Brdiczka (Konstanz, Germany) and developed, finally by ECL, as described [26].

Other methods. CyP-D was purified from rat hearts to a single 20-kDa band on SDS/PAGE exactly as described previously [27]. SDS/PAGE was carried out according to the method of Schägger and von Jagow [28]. Peptidylprolyl *cis-trans*-isomerase (PPIase) activity was measured as described previously [19] at 15°C and using *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl-4-nitroanilide as test peptide.

RESULTS

cDNA cloning and expression of rat CyP-D. There is particularly strong evidence that CyP-D is the CSA 'receptor' of the PT pore ([18–20] and references therein). Accordingly, a CyP-D fusion protein was generated for the identification of CyP-D-binding proteins as candidate constituents of the pore.

Rat CyP-D, including the N-terminal targetting sequence, consists of 206 amino acids [25]. Two independent studies, in which CyP-D isolated from rat liver mitochondria was N-terminally sequenced, showed that residue 30 forms the N-terminus of the mature protein [29, 30]. Residues 1–29 of the nascent protein, therefore, comprise the mitochondrial-targetting sequence which is cleaved off after import into mitochondria to form the mature protein resident in the matrix space. The cDNA isolated in the present study was 5′-truncated, lacking the bases encoding residues 1–13 of the targetting sequence, but it was adequate for the purpose of producing an affinity matrix containing mature CyP-D. This was achieved by cloning the region encoding amino acids 30–206 into pGEX for expression of mature CyP-D as a fusion protein linked to the C-terminus of GST.

Fig. 1 reports expression of the GST–CyP-D fusion protein in XL-1 cells. After induction (Fig. 1, lane b) the 46-kDa fusion

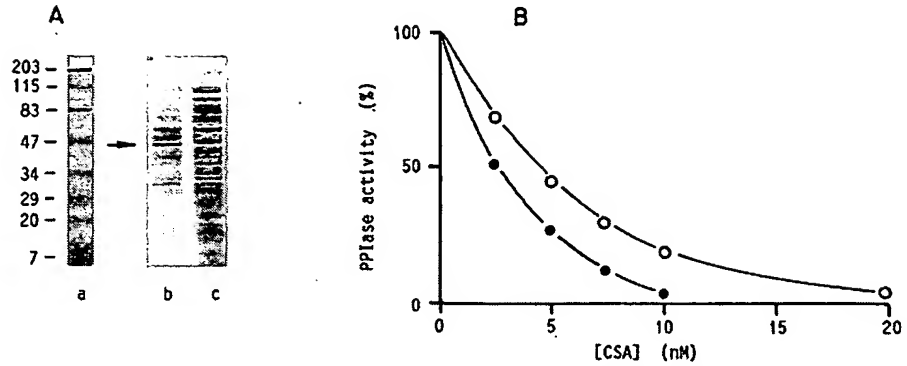

Fig. 1. Expression of a GST/CyP-D fusion protein sensitive to CSA. (A) H_2O-soluble fractions of cells transformed with recombinant pGEX were analysed by SDS/PAGE (Coomassie-blue stained) Lane a, M_r markers. Lane b, induced cells (4 h). Lane c, uninduced cells. (B) The PPIase activity of the purified GST-CyP-D fusion protein (O) and CyP-D isolated from rat heart (●) were compared.

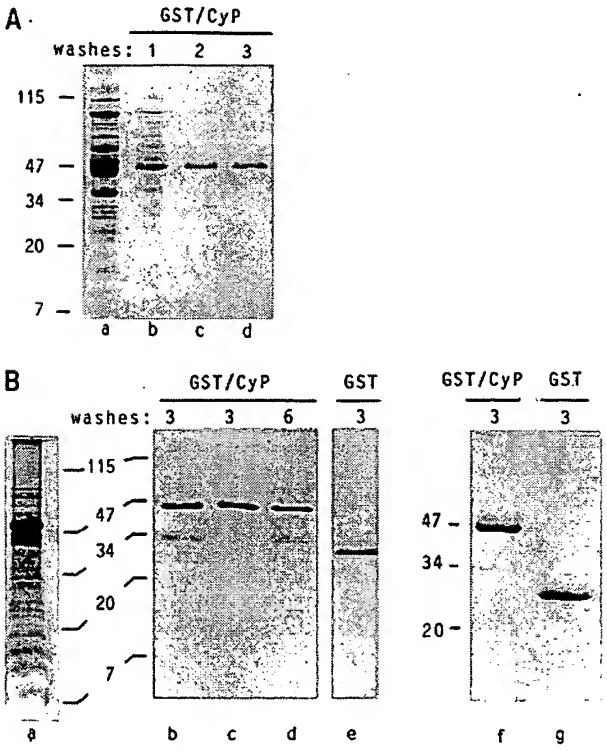

Fig. 2. Binding of a 32-kDa membrane protein(s) by the CyP-D affinity matrix. GST/CyP-D or GST (control) matrices were incubated with mitochondrial extracts and washed 1–6 times as indicated. GST/CyP-D or GST, along with any adherent proteins, were displaced with GSH and analysed by SDS/PAGE. M_r markers, 7–115. (A) H_2O-soluble fraction. Lane a, unfractionated. Lanes b–d, GSH-displaced proteins. Washes contained 0.1 mM EGTA. (B) Membrane fraction. Lane a, unfractionated. Lanes b–g, GSH-displaced proteins. Extracts and washes contained either 0.1 mM EGTA (lanes b–e) or 0.3 mM Ca^{2+} (lanes f and g). Lane c, no membrane extract.

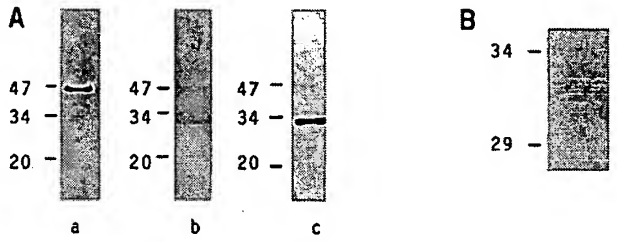

Fig. 3. The 32-kDa protein contains VDAC and ANT. (A) The GST-CyP-D fusion protein and adherent proteins were separated on mini SDS/PAGE as in Fig. 2B, lane b. Lane a, Coomassie-blue-stained gel. Lane b, Western blot developed with anti-ANT polyclonal antibodies. Lane c, Western blot developed with anti-VDAC monoclonal antibody. (B) As in lane a, except on standard (18 cm) SDS/PAGE.

protein was the major cellular protein. The protein was recovered in the soluble fraction of cell sonicates (as in Fig. 1), with no detectable recovery in the sedimentable fraction (10000 *g*, 10 min; not shown).

The fusion protein was purified from the soluble fraction in a single step by binding to GSH-agarose, washing, and elution with GSH, to yield essentially a single 46-kDa protein on SDS/PAGE (e.g. Fig. 2B, lane c). The state of the recombinant CyP-D was assessed from its catalytic activity and from its capacity to bind CSA. PPIase activities were determined using a small chromogenic peptide (see Materials and Methods) at a concentration at least two orders of magnitude lower than the K_m value of cyclophilins for this substrate (about 1 mM) [31]. Semilogarithmic plots of the time courses of isomerization were, therefore, linear (e.g. as in [19]) and were used to derive pseudo-first-order rate constants. The ratio k_{cat}/K_m was $2.4 \pm 0.3\ \mu M^{-1} \times s^{-1}$ (three preparations) at 15 °C. This is similar to the value of $2.9 \pm 0.4\ \mu M^{-1} \times s^{-1}$ (three preparations) obtained for CyP-D purified from rat heart mitochondria, and within the range reported for CyP-A ($1-10\ \mu M^{-1} \times s^{-1}$) [32]. Likewise, the sensitivity of the PPIase activity of the fusion protein to CSA inhibition was comparable to that shown by native CyP-D (Fig. 1B). From this, it appears that the native properties of CyP-D were largely preserved in the fusion protein.

Identification of CyP-D-binding proteins. The affinity matrix comprised the fusion protein bound to GSH-agarose. Extracts were applied, followed by a number of washes, before the fusion protein and any proteins bound to it were displaced by GSH. With the soluble fraction of mitochondria (Fig. 2A) no proteins were retained after two or three washes other than the fusion protein (46 kDa) itself. Similar data were obtained when the extract and wash media contained 0.3 mM $CaCl_2$, in place of EGTA (data not shown). Thus, we obtained no evidence for a H_2O-soluble, mitochondrial protein binding to CyP-D.

With membrane extracts (Fig. 2B), however, a 32-kDa protein was clearly retained after three washes (lane b) and, equally, after six washes (lane d). The 32-kDa band was absent when

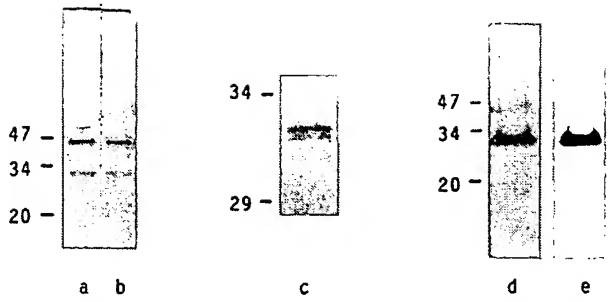

Fig. 4. VDAC and ANT bind to CyP-D in the presence of CSA. A membrane extract was analysed as in Fig. 3 in the absence (lane a) and presence (lanes b–e) of CSA. The matrix was incubated with 40 μM CSA for 15 min before extract, and washes contained CSA (text). Lanes a and b, minigels, Coomassie-blue stained. Lane c, standard gel, Coomassie-blue stained. Lane d, Western blot from minigel, anti-ANT Ig. Lane e, Western blot from minigel, anti-VDAC Ig.

membrane extract was omitted (Fig. 2B, lane c), showing that it originated from mitochondrial membranes. When the standard incubation time of extract with affinity matrix (1 min) was extended (to 10 min, to 40 min) there was no detectable change in the intensity of the 32-kDa band, or the 46-kDa fusion band (not shown), indicating that the 32-kDa band was not a proteolytic product of the fusion protein. As a further control, GST bound to GSH-agarose was used routinely to check any retention of the 32-kDa protein by GST alone. No binding was detected (Fig. 2B, lane e), confirming that the 32-kDa protein bound to the CyP-D moiety of the fusion protein.

Under standard conditions, EGTA was included in the extraction and wash media (e.g. Fig. 2B, lanes b–e). The recovery of the 32-kDa protein was not detectably different when the EGTA was replaced with 0.3 mM $CaCl_2$ (Fig. 2B, lanes f and g). Thus, the binding of the 32-kDa protein to CyP-D was not affected by Ca^{2+}. The significance of this is discussed below.

The 32-kDa band revealed on minigels (6 cm lane length, Fig. 3A) resolved into a doublet on standard length (18 cm) gels (Fig. 3B), and clearly contained more than one component. Regarding their identities, ANT and VDAC have almost the same (subunit) molecular masses of 32 kDa [33, 34], VDAC (in rat) being the slightly larger of the two [35]. Anti-ANT polyclonal antibodies and antiVDAC monoclonal antibodies reacted specifically with the 32-kDa band in Western blots of the fusion protein and the material bound to it (Fig. 3A). The much larger amounts of fusion protein attracted little or no antibody. It may be concluded that CyP-D can associate tightly with VDAC and ANT, most probably in the form of a VDAC/ANT complex.

VDAC is found in the outer membrane, ANT in the inner membrane, whereas CyP-D is localized in the matrix compartment [20]. With this topology, CyP-D would bind to ANT rather than VDAC, and VDAC would be recovered via interaction with ANT. However, there is a huge disparity between the contents of ANT and VDAC in mitochondria. VDAC comprises about 0.3% of the mitochondrial protein [36], which is equivalent to about 100 pmol VDAC/mg protein, or less than 10% the abundance of ANT (1.2 nmol/mg) [33]. On these grounds, one would anticipate that the affinity matrix would bind predominantly ANT, with minor amounts of VDAC. Yet, the data point to significant recoveries of VDAC. It is possible that CyP-D selectively bound to ANT/VDAC complexes, rather than free ANT, under the experimental conditions used. Alternatively, CyP-D might selectively bind to one of the three ANT isoforms; for example, ANT-3 is found at a concentration of 90 pmol/mg in heart mitochondria [33], which is similar to that of VDAC.

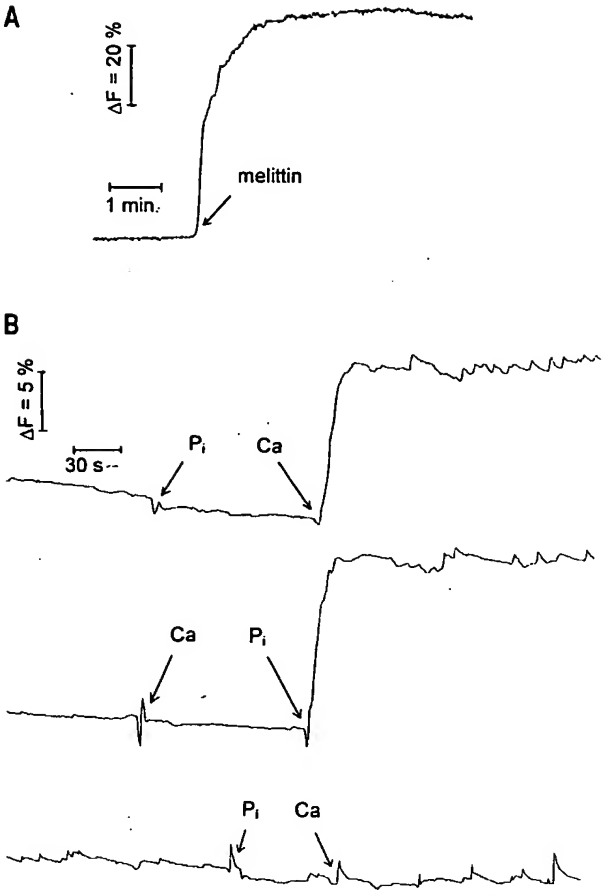

Fig. 5. Reconstitution of pore activity from VDAC, ANT and the CyP-D fusion protein. Proteoliposomes were prepared with the GSH-displaced fraction containing VDAC, ANT and the fusion protein, and loaded with fluorescein sulphonate. (A) 100 nM melittin. (B) 100 μM $CaCl_2$ and 5 mM NaH_2PO_4 added as indicated. In the lowermost trace, the proteoliposomes were incubated with 0.5 μm CSA for 10 min.

A commonly proposed PT pore mechanism (e.g. [18, 19]) envisages that CyP-D binds reversibly to a membrane protein with pore-forming properties as a prerequisite of pore opening. According to this model, Ca^{2+} promotes pore opening by favouring the interaction between CyP-D and the target protein, while CSA inhibits by combining with CyP and preventing the interaction. However, VDAC/ANT was routinely retained by the affinity matrix in the absence of Ca^{2+} (i.e. with EGTA), and the addition of Ca^{2+} did not lead to the retention of further bands (Fig. 2B). Retention in the presence of CSA was also investigated. In this case, the affinity matrix was incubated with 40 μM CSA, in a volume sufficient to give a 10-fold molar excess of CSA over CyP-D, and yield >99.99% saturation of the CSA-binding sites on the affinity matrix (calculated from the measured affinity of GST/CyP-D for CSA; Fig. 1). The conformation of CSA bound to CyP differs markedly from that in solution so that CSA interacts slowly with CyP [37]. For equilibration, therefore, the matrix was incubated with CSA for 15 min, which is quite sufficient to allow equilibration of CSA with CyP-D (3-4 min in control experiments with GST/CyP). With the CSA-saturated matrix, the washes also contained CSA. Yet the CyP-D/CSA matrix retained the 32-kDa band as well as the CyP matrix did (Fig. 4, lanes b and a, respectively) and the VDAC/ANT doublet was just as evident (lanes c–e).

Reconstitution of PT pore activity. The capacity of VDAC, ANT and CyP-D to produce PT pores was tested (Fig. 5). Proteoliposomes were prepared from the VDAC, ANT and the fusion protein displaced from the affinity matrix by GSH (as in Fig. 2B lane d, for example), and loaded with fluorescein sulphonate. The fluorescence of fluorescein is increasingly self-quenched with increasing concentration so that its release from liposomes (i.e. dilution) can be followed fluorimetrically [38]. Fig. 5A is a control experiment in which the proteoliposomes were treated with the pore-forming agent melittin, which permeabilizes bilayers to low M_r solutes [39]. This produced a large increase in fluorescence, indicating fluorescein release. Some vesicles may have contained free VDAC; but if so, they would be expected to be permeant anyway and lack entrapped fluorogen, and go undetected. The proteoliposomes were also permeabilized by Ca^{2+} and phosphate (Fig. 5B, upper and middle) both being necessary. In intact mitochondria, the PT pore is opened by Ca^{2+} plus phosphate, and again both Ca^{2+} and phosphate are essential [2, 7]. Thus, the proteoliposomes exhibited this fundamental property of the permeability transition. The Ca^{2+}-plus-phosphate-induced increase in fluorescence was smaller than that caused by melittin, indicating that only a fraction of the vesicles were responsive to Ca^{2+} plus phosphate; the remainder presumably lacked active (PT pore) proteins. In intact mitochondria, oxidative stress (e.g. peroxides) can substitute for phosphate as co-activator of the PT pore [2]. The proteoliposomes were not permeabilized by Ca^{2+} plus 200 μM *t*-butylhydroperoxide (not shown). However, the mechanism of action of peroxides on the PT pore is obscure i.e. whether it involves oxidised redox coenzymes [1] and/or oxidation of vicinal thiols [3], so that additional components, possibly even proteins, may be required. The most specific characteristic of the PT pore in mitochondria is that it is blocked by CSA [17, 19]. CSA completely inhibited the permeabilization of proteoliposomes by Ca^{2+} plus phosphate (Fig. 5B, lower trace). We conclude, therefore, that PT pore activity had been reconstituted.

DISCUSSION

There is various indirect evidence that VDAC, ANT and CyP-D are constituents of the PT pore (reviewed in [3]). Recently, Ca^{2+}-sensitive and CSA-sensitive pore activities have been reconstituted from fractions containing VDAC, ANT, CyP-D and other mitochondrial proteins [22, 26]. Evidence implicating CyP-D is particularly strong [18–20, 27]. In the present study, CyP-D was found to bind tightly and specifically to a VDAC/ANT complex. No other CyP-D-binding proteins were detected either in the membrane or soluble fractions. These data clearly consolidate the notion of a functional complex between these components. Moreover, the purified proteins produced Ca^{2+}-plus-phosphate-induced pore activity which was blocked by CSA. This is the first demonstration that the three purified proteins generate basic PT pore behaviour, and indicates that the VDAC/ANT/CyP-D complex is the core constituent of the PT pore. Other proteins may associate with the triprotein complex in a regulatory capacity, but the basic pore seems to require only these three proteins. VDAC is found in the outer membrane, whereas ANT is located in the inner membrane, and the detection of a stable VDAC/ANT/CyP-D complex suggests that the PT pore arises at contact sites between the two membranes. We had previously speculated along these lines to explain the complete lack of pore activity in submitochondrial particles (i.e. devoid of outer membranes) supplemented with CyP [19].

CyP-D is H_2O soluble and located in the matrix space [20, 29]. It has been assumed that CyP-D exists in a free state in the matrix and becomes recruited to inner membrane 'pore' sites only under pathological conditions of high matrix Ca^{2+} [18, 19] and/or oxidative stress [40]. However, the VDAC/ANT/CyP-D complex appeared to be stable irrespective of the absence of Ca^{2+} (Fig. 2B) and with the routine inclusion of dithiothreitol (see Materials and Methods) to maintain reduced thiols. Thus, rather than the VDAC/ANT/CyP-D complex being assembled only under extreme pathological states, it seems from the present study that the basic VDAC/ANT/CyP-D complex can exist under far less restrictive conditions (all the time?). Tight binding of CyP-D to the VDAC/ANT complex may explain why CSA-photolabelled CyP-D was routinely recovered in the membrane fraction of mitochondria [20, 27]. It may also explain the electrophysiological detection of single pore activity blocked by CSA in excised patches of inner membrane [41].

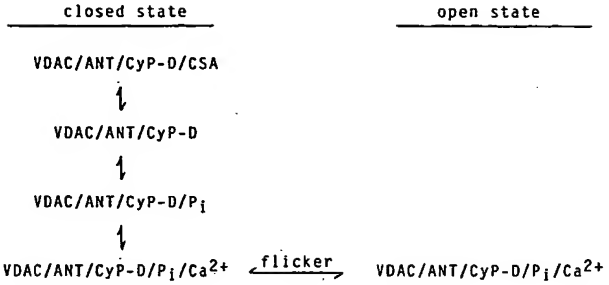

Fig. 6. Suggested model for the PT pore. The pore is assembled at contact sites between VDAC (outer membrane), ANT (inner-membrane) and CyP-D (matrix compartment). The triprotein complex is not disrupted when CSA binds to it. Ca^{2+} and P_i, binding allow flicker into an open pore form.

We were unable to detect any effect of CSA on the binding of VDAC/ANT to CyP-D. Clearly, small changes may have gone undetected with Coomassie-blue staining as measurement, but no gross effect was evident. Of other CyP, CyP-40 binding to heat-shock protein 90 is CSA insensitive, but here the interaction involves the tetratricopeptide repeats in the C-terminal region [42], which are not found in CyP-D. CyP-18 binding to the proline-rich region of $Pr55^{gag}$ in HIV-1 is blocked by CSA [43]. Conversely, the binding of CyP to calcineurin occurs only with CSA, which interacts with both proteins [44]. Thus the CSA-insensitive interaction of CyP-D with a target protein is, to our knowledge, novel and was quite unexpected.

There are two possibilities. The association of VDAC/ANT with CyP-D may occlude the CSA-binding site (i.e. the active site), but be sufficiently strong to compete effectively with CSA (K_d, 2–5 nm; Fig. 1), and allow retention on the CyP-D matrix. In this case, the VDAC/ANT/CyP-D complex, once formed, may undergo a change (PPIase catalyzed?) to a particularly stable state in which CyP-D is practically non-dissociable. There are some data in line with this. Generally, low concentrations of CSA block the pore completely [17]. However, we [19] and others [45] have recorded instances in which pore blockade was incomplete irrespective of [CSA] (e.g. <75% inhibition [19]). Pore resistance to CSA might reflect rearrangements leading to extremely tight binding of CyP-D by VDAC/ANT. The complex between CyP-D and Chaps-solubilized VDAC and ANT may be particularly prone to rearrangement. In contrast, we obtained no evidence for such a stable, CSA-insensitive form of the reconstituted pore, which was completely blocked by CSA (Fig. 5).

Alternatively, VDAC/ANT may bind to a region of CyP-D distinct from the CSA-binding site. There is little conformational change in CyP on binding CSA [37] so that, in principle, CSA need have little effect on VDAC/ANT binding to a non-CSA

region of CyP-D. But equally, according to this model, CyP would relay little information about CSA binding to VDAC/ANT. In other words, CSA would not be expected to cause a major change in the VDAC/ANT conformation, which raises the question of how CSA blocks the pore. Steric effects may be considered. CSA is a cyclic peptide of 11 amino acids (i.e of reasonable size), and it might block by steric hindrance of Ca^{2+} or phosphate binding, or of solute flux if CyP-D is appropriately located near the pore entrance. In agreement with this, following pore opening, CSA blocks PT permeability to relatively large solutes (e.g. sucrose [19]), but not H^+ [46].

The suggested model of the PT pore, in which CyP-D/CSA remains bound to VDAC/ANT, is outlined in Fig. 6. The model incorporates the current finding that Ca^{2+} functions as an allosteric activator, rather than in pore assembly as originally conjectured [18, 19]. Ca^{2+} (together with phosphate) presumably governs the propensity of the pore structure to flicker transiently into an open pore. This agrees with the electrophysiological detection of Ca^{2+}-induced single-pore flicker and its suppression by CSA [5, 41].

The VDAC/ANT/CyP-D complex may have an important role in apoptosis. Release of cytochrome *c* to the cytosol occurs during apoptosis and provides a key step in the activation of caspase-9 (by cytochrome *c*, Apaf-1 and dATP) [47–49]. Cytochrome *c* release is inhibited by overexpression of antiaptotic Bcl-2 [47], and is promoted by overexpression of proapoptotic Bax [50]. The release mechanism may involve rupture of the outer membrane, thus allowing cytochrome *c* and other proapoptotic factors to enter the cytosol [51]. One mechanism proposed for outer membrane rupture involves PT pore opening with consequent expansion of the matrix space [12, 15]. Bcl-2 is an integral outer membrane protein located preferentially at contact sites between the inner and outer membranes [49] and associates with Bax [52]. Thus, Bcl-2 and Bax are suitably located to control apoptosis by interacting with the VDAC/ANT/CyP-D complex forming these contact sites. In turn, the pore-forming capacity of the complex may allow matrix expansion leading to outer membrane rupture and release of cytochrome *c*. Alternatively, by bringing together the inner and outer membranes, the VDAC/ANT/CyP-D complex may serve to facilitate transfer of cytochrome *c* from the inner membrane to an outer membrane system capable of exporting it to the cytosol. The possibility that outer membrane pores assembled from Bax may serve as a cytochrome *c* conduit has been considered [53]. This could occur without PT pore opening and mitochondrial depolarisation and depletion of cellular ATP. The isolation of a VDAC/ANT. CyP-D complex reported here should facilitate studies along these lines.

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